Characterization of Genes Affecting Plant Growth and Development

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Plant development is characterized by the fact that the fate of most cells is not fixed at some relatively early stage during the development of the embryo. Cell differentiation and cell fate determination in most plants are ongoing processes throughout development. As a consequence, most organs consist of cells that are potentially "totipotent", since their fate is not irreversibly fixed. This observation has been well recognized and forms the basis of both experimental and commercial plant tissue culture techniques. Different regimes of so-called growth hormones - such as auxins and cytokinins -, of energy sources (e.g. sucrose) and of various ions can be used to trigger somatic embryogenesis or organogenesis in a wide variety of plant species.

A number of soil bacteria have evolved the capacity to exploit this property of plants to their own advantage. Well known examples are pathogens such as *Agrobacterium tumefaciens*, *A. rhizogenes* and *Pseudomonas savastanoi*.

Indeed these bacteria have evolved mechanisms allowing them to specifically modify the growth and differentiation of various plant organs to suit their own growth requirements. It has been rewarding to study the structure and function of the procaryotic genes that allow these bacteria to specificallly interfere with the "normal" development of their eucaryotic hosts. It was our assumption that the mechanisms by which these procaryotic genes control plant growth and development would be representative of the mechanisms active in the plants themselves. In this, as well as in other ways, these procaryotic genes could be regarded as "oncogenes".

This approach, which was initiated in the early seventies, has thus far provided us with insights in the following mechanisms of control of plant growth and differentiation:

I. Deregulated synthesis of plant growth factors leads to dedifferentiation and tumorous growth

A. tumefaciens induces so-called "crown-gall" tumours on a variety of mostly dicotyledonous host plants. The dedifferentiated growth of the infected tissues is the direct consequence of the deregulated synthesis of auxins and cytokinins by enzymes coded for by three genes (*iaa*M, *iaa*H and *ipt*) carried by a DNA fragment (T-DNA) which is transferred from a plasmid (Ti-plasmid), carried by the pathogenic bacteria, into the genome of the infected plant cells.

II. The activity of growth factors can be modulated by the synthesis of specific antagonists

The T-DNA segments of *A. tumefaciens* strains not only harbour the *iaa*M, *iaa*H and *ipt* genes coding for enzymes catalyzing the synthesis of auxins and cytokinins, but in addition these T-DNAs carry genes, such as gene 5, the function of which is to modulate the activity of the growth factors produced by the major oncogenes.

Indeed, gene 5 was recently shown to be responsible for the synthesis, in transformed plant cells, of an auxin-analogue: indole-lactic acid (ILA). Transgenic tobacco plants expressing gene 5 under control of the 35S RNA promoter of the plant virus CaMV, produce ILA and develop without readily observable alterations. Their seedlings however tolerate levels of exogenously applied auxins that are toxic to isogenic non-transgenic tobacco seedlings. This protection against toxic levels of the growth hormone auxin, might well result from the observation that indole-lactic acid (ILA)

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competes with active auxins such as indole-acetic acid (IAA) for binding to auxin binding proteins that act as auxin-receptors. T-DNAs therefore not only introduce genes in plant cells forcing them to synthesize growth-factors in a deregulated fashion but also introduce a linked gene (gene 5) coding for the synthesis of a growth factor antagonist. The expression of these procaryotic "pathogenesis-genes" in the transformed host cells is fine tuned since it has been shown that the promoter of gene 5 becomes active in the presence of auxins but is repressed in the presence of both auxin and its antagonist ILA.

III. Plant growth factors can not only act extracellularly after transport to target cells but also intracellularly in a cell specific fashion, by activation of intracellular pools of inactive conjugates

Agrobacterium rhizogenes is a pathogen that induces the formation of adventitious roots (called "hairy roots") on a number of plant organs that would not otherwise make such roots. Also, in this case, the abnormal growth was shown to be due to the transfer and expression in plant cells of a set of genes carried on a transferable T-DNA fragment harboured by a plasmid (Ri-plasmid) in *A. rhizogenes*.

The procaryotic genes responsible for the abnormal growth were called *rol* (for root locus). It has been demonstrated that the *rol*B gene, in combination with either the *rol*C gene or the *rol*A gene, was sufficient to induce root growth in several plants and that these genes acted in a cell specific fashion. Indeed, only cells containing these *rol* genes were able to grow as transformed roots. It was therefore thought unlikely that these genes would somehow be involved in the synthesis of growth hormones since these were expected to act extracellularly also on non-transformed cells. In fact it was found that the *rol*C gene codes for an enzyme that releases active cytokinins from inactive intracellular cytokinin glucosides, whereas *rol*B was similarly shown to code for an enzyme capable of hydrolizing inactive auxin-glucosides, thus releasing active auxins in the transformed cells.

IV. Use of gene tagging to identify genes involved in phytohormone perception/ regulation

In order to test whether or not plants normally make use of mechanisms similar to those evolved by these soil bacteria, we initiated a search for plant cell mutants that would be able to grow and differentiate in the absence of extracellularly supplied auxins. In order to rapidly identify and clone genes involved in conveying auxin-independent growth, specially designed T-DNA vectors were used to activate and tag genes that are normally silent in tobacco callus cultures which require auxins for growth. Because the inserted tag is designed to stimulate the transcription of genes, the mutants are expected to be dominant.

At least 4 different classes of dominant auxin-independent mutants were thus obtained. Calli from these mutants grow well in the absence of extracellularly supplied auxins but can be regenerated to form fertile plants. Protoplasts derived from the leaves from these mutants were shown to be able to form calli on media devoid of auxins.

Currently work is underway to characterise the genes responsible for directing auxin independent growth and we are interested in testing whether these mutants will suppress or enhance the abnormal growth of transgenic plants expressing the oncogenes from pathogenic or symbiotic gram negative soil bacteria.

V. Isolation of plant genes encoding auxin binding proteins

A further approach to study the mechanism of action of phytohormones consists in the isolation and functional characterization of potential receptors involved in phytohormone perception and transduction. We have concentrated on the isolation of auxin binding proteins that might act as auxin-receptors. Previous binding and inhibitor studies had shown that plant cells contain different auxin binding sites located in the endoplasmatic reticulum or the tonoplast or the plasma membrane.

An auxin binding protein (ERabp1) was

recently purified and the corresponding genes from *Zea mays* and *Arabidopsis* were cloned and their primary structure deduced. ERabp1 codes for a protein located in the lumen of the ER (with a KDEL sequence at its C-terminus), but also possibly associated with the plasma membrane. Indirect evidence suggests that this auxin binding protein may be a receptor for the auxin signal. Other auxin binding proteins, located at the plasma membrane, may well be involved as influx and efflux carriers in the transport of auxins in plant tissues.

To identify and isolate different auxin binding proteins located on plant cell plasma membranes, we have synthesized auxin-derived photoaffinity probes (5-azido-7-^{3}H-indol-3-acetic acid) to covalently label auxin binding proteins. Three different proteins were labelled called pm23, p58 and p60.

p60 is apparently a member of a gene family and might well be synthesized as a larger precursor protein having a N-terminal extension. The predicted amino acid sequence of a cDNA clone that corresponds to a p60 protein shares peptide homology with a number of eucaryotic ß-D-glucosidases. Indeed, p60 was shown to have ß-D-glucoside glucohydrolase activity (E.C.3.2.1.21). Interestingly a conserved central region of p60 shares similarity with a region of the *A. rhizogenes rol*C gene which encodes a cytokinin-N-glucoside glucosidase. Preliminary evidence indeed suggests that p60 can hydrolyze cytokinin-glucosides. Whereas p60-like proteins were detected both in plasma membrane enriched and cytosolic fractions, this is apparently not the case for pm23 which was detected only in fractions highly enriched for plasma membranes. Its labeling by 5-azido-IAA was efficiently and specifically inhibited by TIBA (2,3,5 triiodo-benzoic acid), a potent polar auxin transport inhibitor and by the phytotropin naphthylphthalamic acid (NPA) indicating that pm23 could well be part of an auxin efflux carrier. A cDNA clone corresponding to pm23 from maize coleoptiles was isolated and sequenced. No extensive homology to ERabp1 and no similarity to sequences in data bases were detected.

The p58 protein has not been thus far subjected to further studies.

In summary: the extensive analysis of auxin binding proteins has not revealed any typical eucaryotic transmembrane receptor protein. The question therefore arises whether such classical receptors are involved in auxin perception and response.

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